Abstract

Inhibitory effect of *Lactobacillus* spp. isolated from healthy Korean women on vaginal pathogens

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Lactobacilli play an important role in vaginal health through production of byproducts such as lactic acid, hydrogen peroxide (H_2O_2) and bacteroicins. The prevalence of vaginal pathogens as well as the depletion of lactobacilli are both associated with numerous adverse health outcomes including bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC). Among the BV candidate bacterial species, *Gardnerella vaginalis*, a well-known vaginal pathogen, and *Sneathia*

species are recently emerging as opportunistic pathogens associated with a variety of conditions associated with vaginal infections such as preterm labor, spontaneous abortion, and HPV infection. In this study, we investigated the antimicrobial activities of the supernatant of 51 *Lactobacillus* strains isolated from 3 healthy subjects on *Gardnerella vaginalis, Sneathia sanguinegens* (BV pathogens) and *Candida albicans* (VVC pathogen). Also, we assessed the association between the activities and levels of supernatant pH, D- and L-lactate, and production of hydrogen peroxide. As a results, the tested *Lactobacillus* isolates were clustered by inhibition patterns against BV and VVC pathogens into four clusters (Cluster Ⅰ~Ⅳ). *Lactobacillus* isolates belonging to Cluster Ⅳ showed the greatest inhibitory activities to all tested pathogens and significantly higher levels of D-/L-lactate ratio than the other clusters (P <0.0001). To clarify the association between production of lactic acid isomers and pathogen inhibitory effect, we conducted correlation analysis. D-lactate showed significant negative association with the growth rate of all of vaginal pathogens, but no vaginal pathogens associated with L-lactate. Also, to identify the origin specificity of *Lactobacillus* in terms of lactate isomer producing, we compared the lactate producing patterns of vagina- and gut-originated strains. As a result, we identified that D-lactate was produced by

lactobacilli in a species- and origin-specific manner and that D-lactate determines inhibitory patterns of human vaginal lactobacilli isolates on different vaginal pathogens.

Key words: *Lactobacillus*, Bacterial vaginosis, *Gardenrella vaginalis*, *Sneathia sanguinegens, Candida albians,* vaginal infection

Student No. 2015-24056

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I. Introduction

Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are two of the most prevalent vaginal infections in women. Bacterial vaginosis is the most common disorder of vaginal flora (1) and is caused by the reduction of lactobacilli in the vagina and subsequent replacement by a mixed microflora dominated by anaerobes (2). BV is also defined by a clearance of lactobacilli and an increase in Gram-variable and Gram-negative rods (3). *Gardnerella vaginalis*, as well as *Adopobium vaginae, Bacteroides, Prevotella*, and *Mobiluncus* species are representative bacteria of those increased groups (4-6). *Sneathia* species are other BV candidate bacteria that are recently emerging as potential pathogens associated with preeclampsia, preterm labor, spontaneous abortion and HPV infection as well as BV (7, 8). Approximately fifty percent of women will experience a BV infection at some point in their lifetime, with 1,080,000 cases occurring per year in pregnant women in the United States (9, 10). Vulvovaginal candidiasis is a major vaginal yeast infection that is often caused by *Candida albicans* (11) and almost three quarters of women will have an episode of VVC at least once in their lifetime (12). BV and VVC are associated with a variety of clinical symptoms, including a thin, white or yellow discharge, a fishy smell

and increased susceptibility to vaginal infections and sexually transmitted disease including HIV (13-16). During pregnancy, BV and VVC also increase the risk of preterm labor and delivering a low birth-weight infants (17-19).

The microbiota of the vagina perform an important role in the maintenance of vaginal health and the prevention of infection. Healthy vaginal microbiota are generally dominated by one or more species of *Lactobacillus*, the most common of which are *Lactobacillus crispatus, L. jensenii, L. iners, L. gasseri*, and *L. fermentum*. (20-22). Lactobacilli maintain a low pH environment in the vagina by producing lactic acid, hydrogen peroxide and further protect the host from urogenital infection through the production of bacteriocins (23-26).

Lactic acid is a primary metabolite produced by lactobacilli. Lactic acid producing bacteria including lactobacilli produce both the D- and L-lactic acid isomers (27), while mammalian cells produce only the L-lactic acid isomer except for the case of base line production of the D-lactic acid isomer via the methylglyoxal pathway (28). In recent studies, the ratio of D- to L-lactic acid in vaginal specimens differed based on the dominated *Lactobacillus* species and the ratio influenced the regulation of an enzyme involved in upper genital tract infection, suggesting a relationships between the amount of L-lactic acid in the

vagina and upper genital tract infection (29-31). However, neither the role that lactic acid isomers play in directly affecting vaginal pathogens or the correlation between lactic acid productivity and inhibitory effects have yet to be investigated. In this paper, we investigated the concentration of each lactic acid isomer produced by *Lactobacillus* strains and subsequently compared the concentration and the effect that each *Lactobacillus* strains had on inhibiting the growth of BV and VVC pathogens. We also assessed the association between the growth inhibition of BV and VVC pathogens and the *Lactobacillus* supernatant characteristics including supernatant pH level, presence of D- and L-lactic acid, and hydrogen peroxide production. *G. vaginalis* KCTC 5096 and *S. sanguinegens* isolated from the vagina were tested as BV pathogens and *C. albicans* ATCC MYA4788 and *C. albicans* ATCC 44858 were used as VVC pathogens.

II. Materials and Methods

1. *Lactobacillus* strains and culture

Lactobacillus strains isolated from the human vagina and gut were used in this study (Table 1). *Lactobacillus* strains isolated from vaginal specimens obtained from a healthy Korean women trio consisting of a monozygotic twin pair and their mother were used to test the antimicrobial capability of each *Lactobacillus* strain against vaginal pathogens. Strains originating from the gut were used to compare their lactate productivity or carbohydrate fermentation capacity with their vaginal strain counterparts. All lactobacilli were cultured in MRS broth with 0.05 % L-cysteine hydrochloride (Sigma-Aldrich) anaerobically at 37 ℃ for 24 h. The cultural broths were centrifuged at 13,000 rpm for 5 min and the supernatants were then filter-sterilized using 0.22 μm pore size syringe filter units (Millipore Co., Italy) to remove any remaining bacterial cells and stored at -20 ℃ for analysis.

2. Vaginal pathogens and culture

Microorganisms related to BV or VVC were used in this study as vaginal pathogens for evaluating the antimicrobial activities of lactobacilli. *G. vaginalis* and *S. sanguinegens* were used as representative bacteria related to BV and *C. albicans* was the representative microorganism for VVC associated yeast.

G. vaginalis KCTC 5096 was obtained from the Korean Collection for Type Cultures (Daejeon, Korea) and *S. sanguinegens* was isolated from the human vagina. *G. vaginalis* and *S. sanguinegens* were grown in ATCC medium 1685 (NYC Ⅲ medium) containing 5 % horse serum at 37 ℃ for 18 h using Rectangular jar containing Anaero-Pack (Mitsubishi gas chemical, Japan).

Candida albicans ATCC MYA4788 and ATCC 44858 were purchased from American Type Culture Collection and cultured aerobically in YM broth at 37 ℃ for 18 h in a shaking incubator. A subculture was prepared for later co-culturing with *Lactobacillus* cell free supernatant (CFS).

3. Characterization of *Lactobacillus* spp. by pH and the production of hydrogen peroxide

Acidity of CFS of *Lactobacillus* isolated from the vagina was measured using benchtop pH meter (Thermo Scientific, USA). *Lactobacillus* strains were tested to evaluate their ability to produce H_2O_2 as described by Rabe and Hillier (32) with slight modifications. All of strains were cultured onto MRS agar plates containing 25 mg 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich), 0.5 mg hemin and 0.05 μg vitamin K (Sigma-Aldrich) (33). The plates were incubated anaerobically at 37 ℃ for 48 h. After incubation, the plates were exposed to air for 30 min to check for a blue color change.

4. Characterization of *Lactobacillus* spp. by the production of lactate

To quantitatively determine the lactic acid isomers produced by each *Lactobacillus* stain, EnzyChrom L- and D-lactate kits (BioAssay Systems, Hayward, CA) were used. *Lactobacillus* strains isolated from the vagina or gut were cultured in MRS broth medium and subsequently filter-sterilized. Lactate concentration in each CFS was measured by the colorimetrical method.

5. Test of *Lactobacillus* spp. for bile acid tolerance

To measure the relative growth rate of lactobacilli in bile containing media, filter-sterilized solutions of commercial bile acid mixture (Bile from bovine and ovine, Sigma-Aldrich) were added to autoclaved MRS broth at different concentrations (control, 0.1 %, 0.5 %, 1.0 %, 2.0 % and 4.0 %). Each *Lactobacillus* strain was inoculated (1 % v/v) in six different conditions and incubated for 24 h in a 96 well plate (34). After 24h, bile acid tolerance identified through measuring the absorbance at 600 nm and comparing to a control. The relative growth rate in the response to bile acid presence was expressed as bile acid tolerance (35).

6. Test of *Lactobacillus* spp. for acid tolerance

Each strain of *Lactobacillus* isolates was inoculated into MRS broth acidified with concentrated hydrochloric acid to pH 2, 3 or pH6.8 (non-acidified) and incubated at 37 ℃ for 24 h. After incubation, acid tolerance identified through measuring the number of viable colony counts.

7. Test of *Lactobacillus* spp. for antimicrobial activity against vaginal pathogens

G. vaginalis and *S. sanguinegens* were grown anaerobically in NYC Ⅲ broth and *C. albicans* strains were cultured aerobically in YM broth at 37 ℃. The antimicrobial activities of *Lactobacillus* CFS against vaginal pathogens were tested as described by Toba *et al*. (36). Briefly, Pathogen suspensions were prepared in fresh medium from overnight cultures and the turbidity was adjusted to OD_{600} =0.1. Each well of a 96 well plate was inoculated with 200 μl of a pathogen suspension, and subsequently 100 μl of *Lactobacillus* CFS was added to each well. The growth control well contained 100 μl of sterile MRS medium and 200 μl of same pathogen suspension. The 96 well plates were incubated at 37 ℃ and growth was measured after 24 h. The results were read in turbidity relative to the control by measuring the absorbance at 600 nm with Infinite M200 Microplate Reader (TECAN, Switzerland). To determine the effect of lactic acid on pathogenic growth inhibition, following the same methods, L- and D-lactic acid antimicrobial activities were tested for all pathogens.

8. Characterization of the substrate metabolizing capacity of *Lactobacillus* spp.

Carbohydrate fermentation profiles of lactobacilli were tested by commercial kit API 50 CHL (BioMerieux, France), which consists of 49 biochemical tests for the study of carbohydrate metabolism of lactobacilli. Overnight cultures of *Lactobacillus* were diluted with API 50 CHL media to adjust OD value to McFarland 2 and inoculated in each substrate containing well. After incubation for 48 h, fermentation profiles were identified through the API identification software database (https://apiweb.biomerieux.com) based on the media color of each well.

9. Statistical analysis

All data were analyzed with Prism 5 (GraphPad software, San Diego, CA) or R studio (https://www.rstudio.com/). Clustering analysis was performed using the pheatmap package in R. The distance within *Lactobacillus* strains was calculated using the Euclidean method when comparing the inhibitory patterns or fermentation profile among lactobacilli. Statistical significance was measured using the Mann-Whitney test for comparing two groups and the Kruskal-Wallis test with Dunn's multiple comparison test for comparison of three or more groups. In graphical representations, data were presented as mean ± standard error mean. The whiskers of each boxplot represent the 10 to 90 percentiles. Statistical significance was given as * P-value <0.05, ** P-value<0.01, *** P-value<0.001.

III. Results

1. Profile of vaginal *Lactobacillus* strains

Fifty-one *Lactobacillus* isolates that were obtained from vaginal swabs of three healthy Korean women (M, T1 and T2) with no specific urogenital diseases including BV or VVC were used in this study. All isolates were identified to the species level by sequencing the 16S rRNA before the assay. The *Lactobacillus* isolates consisted of fifteen *L. crispatus*, thirteen *L. fermentum*, seven *L. gasseri* and sixteen *L. jensenii* strains (Table 1). The total number of *Lactobacillus* isolates per subject was 6, 23 and 22 strains each. Although subject T1 and T2 were a pair of monozygotic twins having nearly identical DNA and subject M is their mother, they displayed unique distributions of *Lactobacillus* species in their vaginal specimens based on the results of their cultured samples. The dominant species based on the number of isolates are *L. crispatus* (83.3 %), *L. fermentum* (56.5 %) and *L. jensenii* (68.2 %) for M, T1 and T2 respectively (Figure 1).

Table 1. *Lactobacillus* strains used in this study

Lactobacillus species	Isolated from vagina		Isolated from gut	
	strain	No. of isolates	strain	No. of isolates
Lactobacillus crispatus	SNUV152 SNUV210 SNUV219 SNUV220 SNUV227 SNUV236 SNUV241 SNUV250 SNUV253 SNUV282 SNUV358 SNUV367 SNUV381 SNUV385 SNUV476	15		-
Lactobacillus fermentum	SNUV155 SNUV157 SNUV168 SNUV175 SNUV240 SNUV245 SNUV257 SNUV258 SNUV259 SNUV264 SNUV374 SNUV430 SNUV444	13	SNUG60013 SNUG60059	2
Lactobacillus gasseri	SNUV159 SNUV281 SNUV431 SNUV457 SNUV462 SNUV463 SNUV464	7	SNUG50019 SNUG50077 SNUG50243 SNUG50390 SNUG50417 SNUG50579 SNUG60024 SNUG60080 SNUG60134	9
Lactobacillus jensenii *Lactobacillus jensenii* (continued)	SNUV181 SNUV184 SNUV185 SNUV190 SNUV200 SNUV226 SNUV287 SNUV290 SNUV291 SNUV354 SNUV360 SNUV448 SNUV465 SNUV470 SNUV478 SNUV479	16		-
Lactobacillus paracasei subsp. *tolerans*		-	SNUG50501	1
Lactobacillus plantarum subsp. *plantarum*		-	SNUG10271	1

Table 1 *(continued)*. *Lactobacillus* strains used in this study

Lactobacillus species	Isolated from vagina		Isolated from gut	
	strain	No. of isolates	strain	No. of isolates
Lactobacillus reuteri		-	SNUG50382	1
Lactobacillus rhamnosus			SNUG50057 SNUG50070 SNUG50362 SNUG50415 SNUG50461 SNUG50545	6
Lactobacillus ruminis		-	SNUG30003 SNUG30020 SNUG60001 SNUG60032 SNUG60081 SNUG60098 SNUG60127 SNUG60132	8
Lactobacillus salivarius		-	SNUG10354 SNUG60058 SNUG60106 SNUG60122 SNUG60133	5
Total		**51**		**33**

Fifty-one vaginal *Lactobacillus* strains and thirty-three strains isolated from the gut were used in this study.

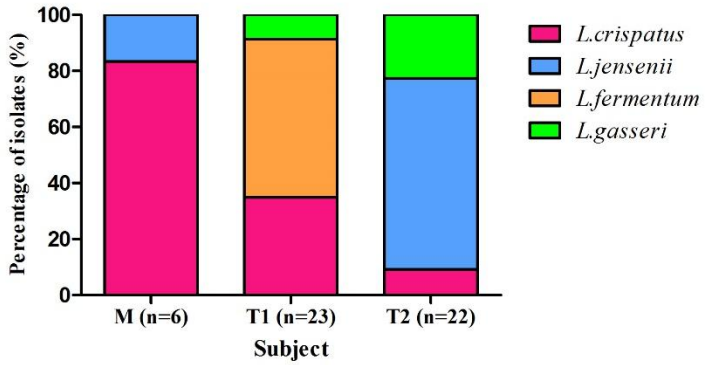


Figure 1. Distribution of *Lactobacillus* species isolated from the vagina of each subject

Fifty-one *Lactobacillus* strains were isolated from three healthy Korean women, which consisted of a pair of monozygotic twins (T1 and T2) and their mother. Different colors express the percentage of isolates belonging to the different species in each subject. Along the x-axis, the total number of isolates per subject is indicated in parentheses.

2. Acidity and hydrogen peroxide productivity of *Lactobacillus* spp. isolated from the vagina

We evaluated the pH and hydrogen peroxide productivity of vagina-originated *Lactobacillus* strains. The pH of lactobacilli supernatant was measured to be in the range 3.88 – 4.75, showing the characteristics of all strains to acidify the culture medium. *L. crispatus* SNUV210 had the lowest pH value (pH 3.88), acidifying the medium strongly, and was the only strain measured to have a pH lower than 4.0. At the species level, *L. fermentum* reduced supernatant pH values significantly lower than *L. jensenii* ($P < 0.05$) (Figure 2A). No statistically significant differences were detected between other species. On the other hand, among all of the tested vaginal *Lactobacillus* strains, 9 strains produced H_2O_2. Two of them belonged to *L. fermentum* and *L. gasseri* and the remaining strains were *L. jensenii* species. Interestingly, all *L. crispatus* species used in this study were H_2O_2-negative and most of H_2O_2-positive strains were associated with *L. jensenii* species (Figure 2B).

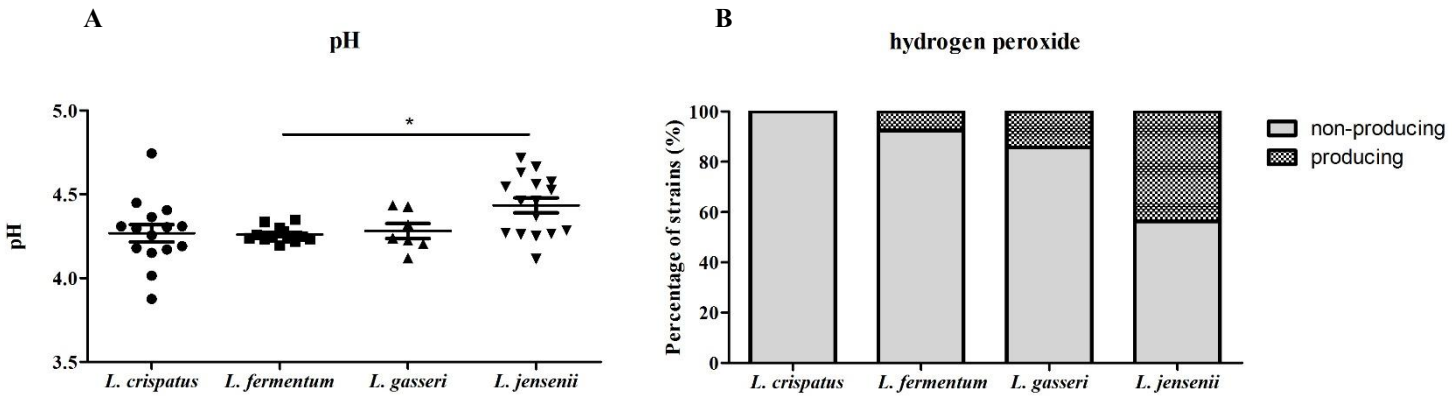


Figure 2. pH and H_2O_2 production of *Lactobacillus* spp. isolated from the vagina

To determine the characteristics of lactobacilli by species, supernatant pH and hydrogen peroxide productivity were measured. (A) The pH of the filter-sterilized cell-free supernatant (CFS) was measured using a benchtop pH meter. (B) Hydrogen peroxide productivity was measured on TMB agar qualitativly and expressed as the number of producing or non-producing strains in stacked bar graph. The results were analyzed by the Kruskal-Wallis test with Dunn's multiple comparison test. * $P < 0.05$

3. Lactate productivity of *Lactobacillus* spp. isolated from the vagina

D-lactate was produced by all isolates at concentrations ranging from 37.05 – 128.3 mM. The mean concentration of D-lactate was higher at *L. crispatus* than other *Lactobacillus* species, with *L. crispatus* SNUV220 bring the strain showing the highest production of D-lactate (Figure 3A). However, due perhaps to the broad concentration range, there was no measurable significant differences among the species. L-lactate production also did not seem related to a particular species, but 3 strains of *L. jensenii* (SNUV354, SNUV360 and SNUV448) were poor producers, producing less than 2.0 mM respectively (Figure 3B). Meanwhile, the ratio of D- to L-lactate was significantly different between *L. crispatus* and *L. fermentum* ($P < 0.05$). Although they have no differences between concentration of D- and L-lactate, the *L. crispatus* species contained the strains that produce more D-lactate than L-lactate (Figure 3C).

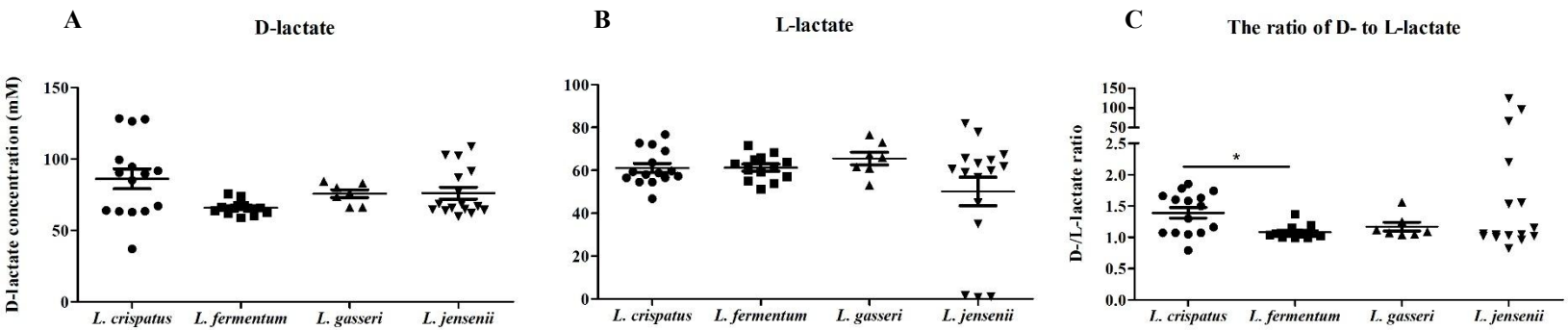


Figure 3. Lactate concentration produced by *Lactobacillus* spp. isolated from the vagina

To determine the production of lactate isomers by each species, the concentration of each lactate isomer was measured using the colorimetric method. (A) D-lactate and (B) L-lactate were assessed in *Lactobacillus* CFS quantitatively. (C) The ratio of D- to L-lactate were simply calculated by dividing the D-lactate concentration by the L-lactate concentration. Results were analyzed by the Kruskal-Wallis test with Dunn's multiple comparison test. * $P < 0.05$

4. Bile acid- and acid-tolerance of *Lactobacillus* spp. isolated from the vagina

Bile acid sensitivity was defined as the decrease in the relative growth rate in response to bile acid presence in the growth media. In general, *Lactobacillus* growth was decreased proportionately to the bile salt concentration. The majority of the strains were resistant to 0.1 % bile acid showing over 60 % of growth rate with the exception of *L. crispatus* SNUV367, *L. gasseri* SNUV462, *L. jensenii* SNUV290, SNUV291, SNUV465 and SNUV470 (Figure 4E). However, at concentrations over 0.5 %, the growth rate of lactobacilli showed a broad range of tolerance by species and strain. In the media containing all different concentrations of bile acid, *L. fermentum* showed a significantly higher growth rate than *L. crispatus* or *L. jensenii*. Even in the 0.5 % bile acid containing media, *L. fermentum* strains significantly higher growth rates than the other species (Figure 4). When *Lactobacillus* strains were cultured in MRS medium acidified to pH 3, the growth rate of most of strains was also decreased. Result of the spearman correlation analysis between bile acid- and acid-tolerance was shown in Figure 5. The relative growth rate of *Lactobacillus* strains that exposed to the acidified media showed significant correlation with bile acid tolerance.

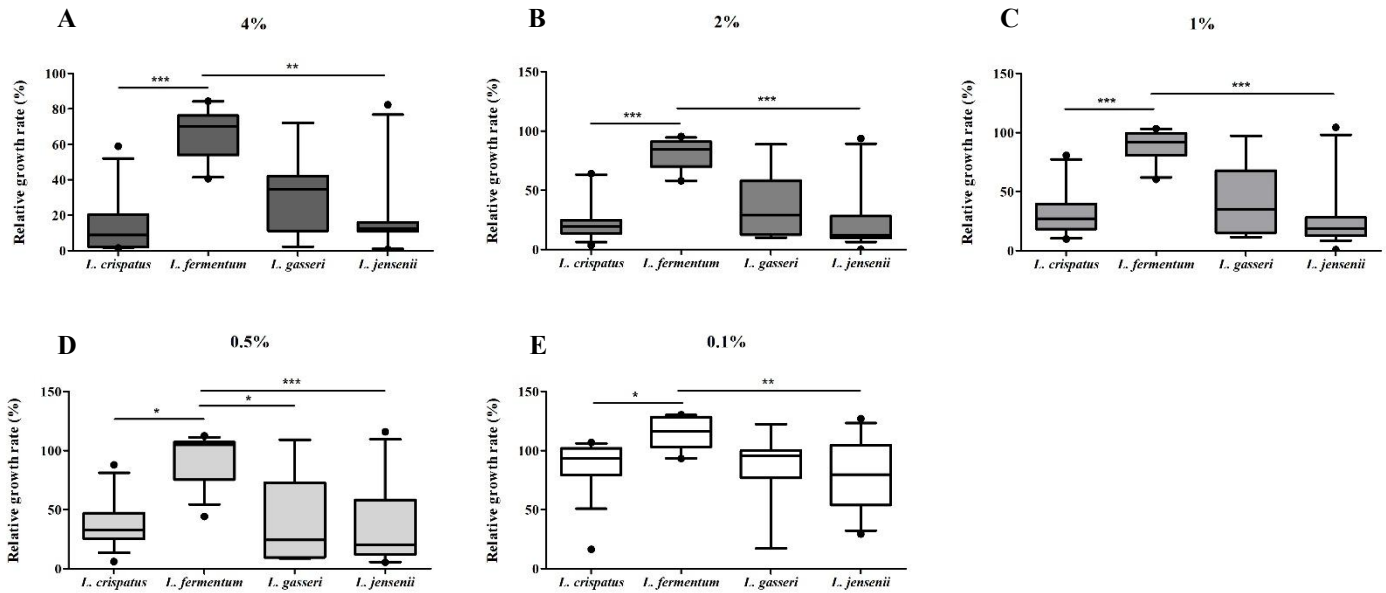
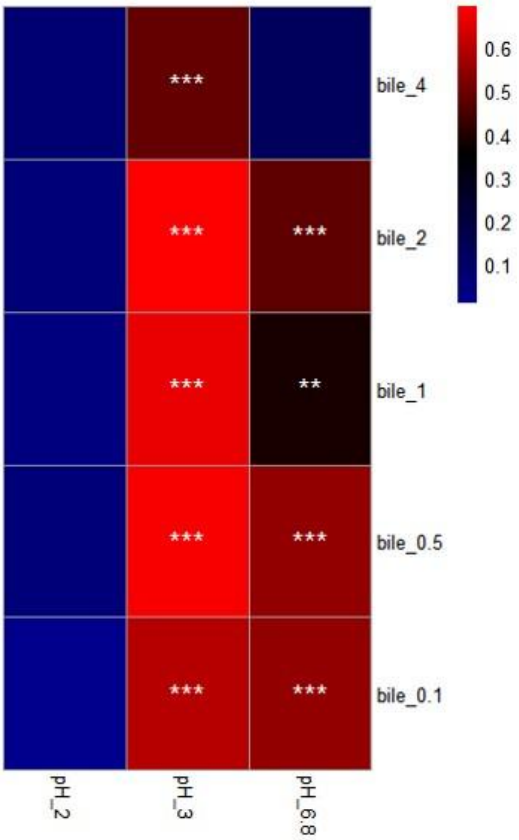


Figure 4. Bile acid tolerance of *Lactobacillus* spp. isolated from the vagina

To compare the tolerance to bile acid among species, lactobacilli were cultured in bile containing MRS medium and the growth rate was assessed based on the absorbance at 600 nm. The relative growth rate is expressed as the ratio of OD_{600} with each respective bile acid concentration (A; 4 %, B; 2%, C; 1%, D; 0.5% and E; 0.1% bile acid) and control (no bile acid). Experiments were carried out in triplicate. The whiskers of each boxplot represent the 10 to 90 percentiles. Results were analyzed by the Kruskal-Wallis test with Dunn's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 5. The correlation between bile acid- and acid-tolerance of *Lactobacillus* spp. isolated from the vagina



Spearman correlation analysis was performed to analyze the correlation of the tolerance to bile acid (4 %, 2 %, 1 %, 0.5 % and 0.1 % of bile slats) and acid (pH 2, 3 and 6.8). The relative growth rate of *Lactobacillu*s strains that exposed to acidified environment was compared with the relative growth rate of *Lactobacillus* strains cultured in the bile containing medium. Color gradation represents the Spearman *r*. ** $P < 0.01$, *** $P < 0.001$

5. Antimicrobial activity of *Lactobacillus* spp. isolated from the vagina on vaginal pathogens

The antimicrobial activities of the CFS of the vaginal *Lactobacillus* isolates were evaluated against *G. vaginalis*, *S. sanguinegens* and *C. albicans*. All *Lactobacillus* strains suppressed the growth rate of BV or VVC pathogens differently (Figure S1). To compare and characterize the inhibitory effect of lactobacilli against each pathogens, we calculated the Euclidean distance between *Lactobacillus* strains based on their inhibitory effect against the pathogens. The distances were ranging from 1 to 48.6. Based on the Euclidean distance 12.5, *Lactobacillus* strains were divided into four clusters that were characterized by their different pathogenic inhibition characteristics (Figure 6A). The strains included in each cluster are shown in Table S1. The growth rate of the vaginal pathogens treated by each *Lactobacillus* strain are indicated in color gradation. The species and subject of each isolates are also presented as different colors. The strains belonging to Cluster Ⅰ or Ⅱ inhibit BV or VVC pathogens respectively. Meanwhile, the strains belonging to Cluster Ⅲ and Ⅳ showed inhibitory effects against all pathogens. Especially, Cluster Ⅳ which was comprised of the most effective strains seemed to be related with *L.*

crispatus and *L. jensenii*.

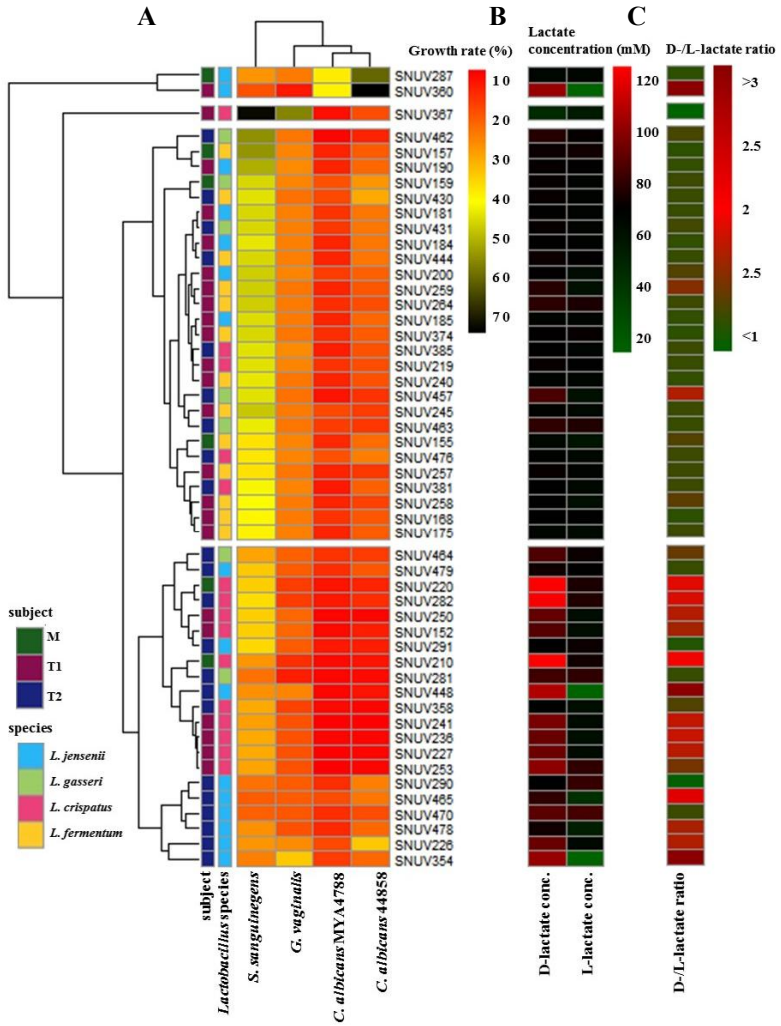


Figure 6. The patterns of the capacity of pathogen inhibition and lactate production of *Lactobacillus* spp. isolated from the vagina

To compare the inhibitory patterns on different vaginal pathogens and lactate productivity among lactobacilli, *Lactobacillus* strains were listed by their inhibition capacity. (A) The heatmap was generated based on the growth rate of vaginal pathogens. The trees were drawn using the Euclidean method and the lactobacilli were divided into four clusters. (B) The concentration of D- and L-lactate produced by each *Lactobacillus* strain and (C) D-/ L-lactate ratios were also expressed as heatmaps. Each color gradation represents the growth rate (%), concentration (mM) or ratio of concentration.

6. The comparison between antimicrobial activity and the capacity to produce lactate isomers

The concentration of lactate isomers produced by each *Lactobacillus* strain and the ratio of isomers were also presented as color gradation in heatmaps (Figure 6B, C).

After comparison of the inhibitory effect of lactobacilli and the lactate concentration contained in their CFS, all lactobacilli belonging to Cluster Ⅲ or Cluster Ⅳ were compared in respect to their concentrations of produced D- and L-lactate and the ratio of D- to L-lactate (Figure 7A, D). The strains in Cluster Ⅳ that showed strong effects on all vaginal pathogens produced significantly more D-lactate than strains in Cluster Ⅲ ($P < 0.0001$, Figure 7A). D-/L-lactate ratios also were in concordance with the concentration of D-lactate ($P < 0.0001$, Figure 7D). To confirm that the differences in D-lactate concentration and D-/L-lactate ratio between clusters were associated with the different inhibitory patterns, rather than the different composition of the species, the comparison was also conducted at the species level. Because *L. crispatus* and *L. jensenii* were common species in both clusters, lactate isomer concentration and the ratio of lactate isomers produced by *L. crispatus* and *L. jensenii* were compared

between clusters. The comparison of the concentration of D-lactate showed that both species were significantly different ($P < 0.05$, Figure 7B~C), but in the case of D-/L-lactate ratio, only *L. crispatus* showed the significant difference ($P < 0.01$, Figure 7E). Although *L. gasseri* was also common between the clusters, the species was not selected for comparison analysis due to the small number of strains included in Cluster Ⅳ. In all comparisons, there was no significant difference in L-lactate (data not shown)

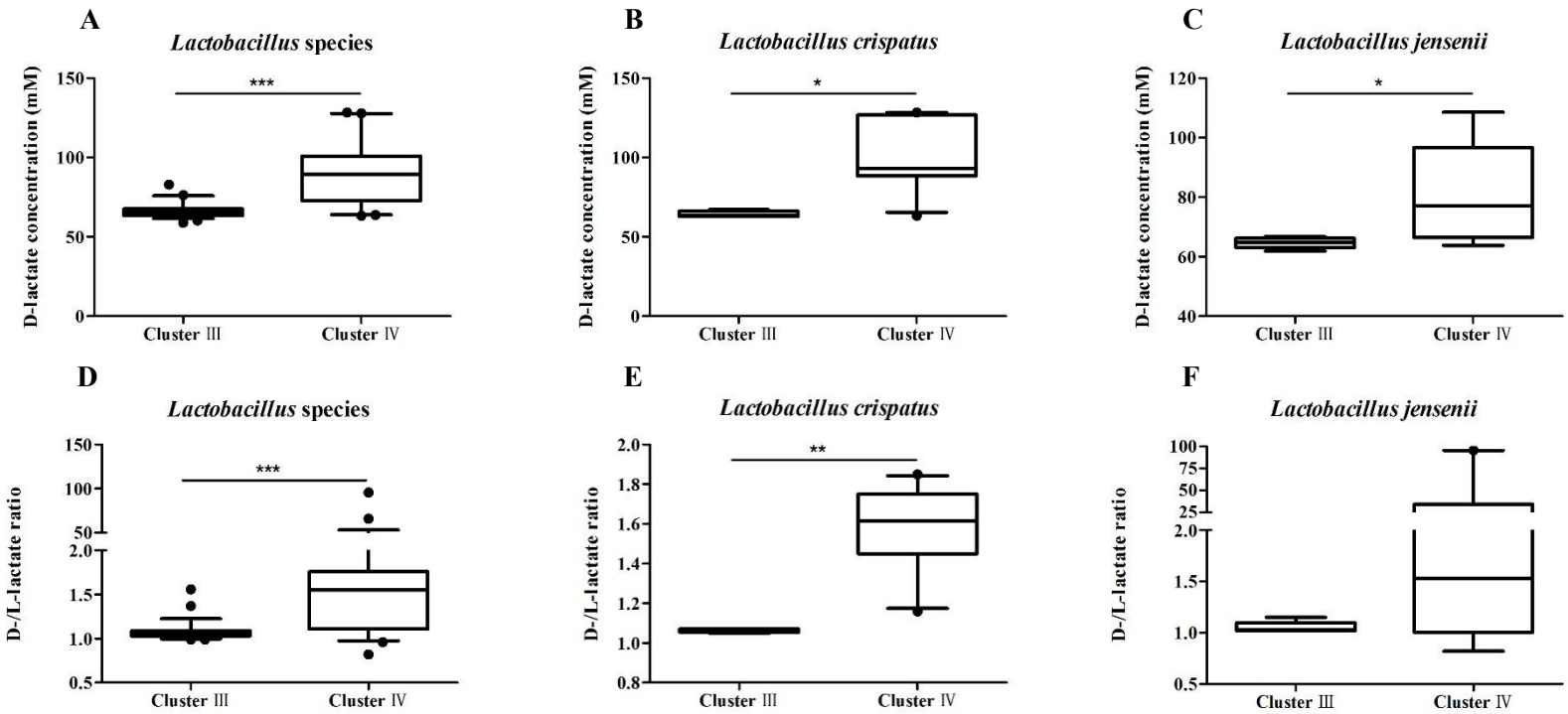


Figure 7. Lactate production of *Lactobacillus* spp. isolated from the vagina in inhibition cluster Ⅲ and Ⅳ

Lactate concentration produced by lactobacilli were compared between the two clusters. The whiskers of each boxplot represent the 10 to 90 percentiles. Statistical significance for the difference between isomers affecting growth was analyzed by the Mann-Whitney test. * P<0.05, ** P<0.01, *** P<0.001

7. The correlation between antimicrobial activity and the characteristics of *Lactobacillus* spp. isolated from the vagina

To clarify the relationships between inhibitory effect and the characteristics of *Lactobacillus* strains, including their pH levels, H_2O_2 production, and each lactate isomer concentration, we conducted correlation analysis. The correlation between inhibitory effect and the levels of D- or L-lactate isomer are shown in Figure 8. The concentration of D-lactate showed negative statistical correlation to the growth rate of all tested vaginal pathogens, *G. vaginalis* (Spearman r = -0.4669, P = 0.0006, Figure 8A), *S. sanguinegens* (Spearman r = -0.453, P = 0.0008, Figure 8B) and *C. albicans* ATCC MYA4788 (Spearman r =-0.2929, P = 0.037, Figure 8C) and ATCC 44858 (Spearman r = -0.4127, P = 0.0026, Figure 8D). The ratio of d- to l-lactate also negatively correlated with the growth rate of *G. vaginalis* (Spearman r = -0.3399, P = 0.0147), *S. sanguinegens* (Spearman r = -0.4588, P = 0.0007), and C. *albicans* ATCC 44858 (Spearman r = -0.3035, P = 0.0304) but ATCC MYA4788 (Spearman r = -0.22, P = 0.1208) was not significant. Meanwhile, there was no significant correlation between the growth rate of vaginal pathogens and L-lactate concentration (Figure 8E~H). pH and H_2O_2 production were also analyzed for correlation, but they each showed no association with the growth rate

of vaginal pathogens (data not shown).

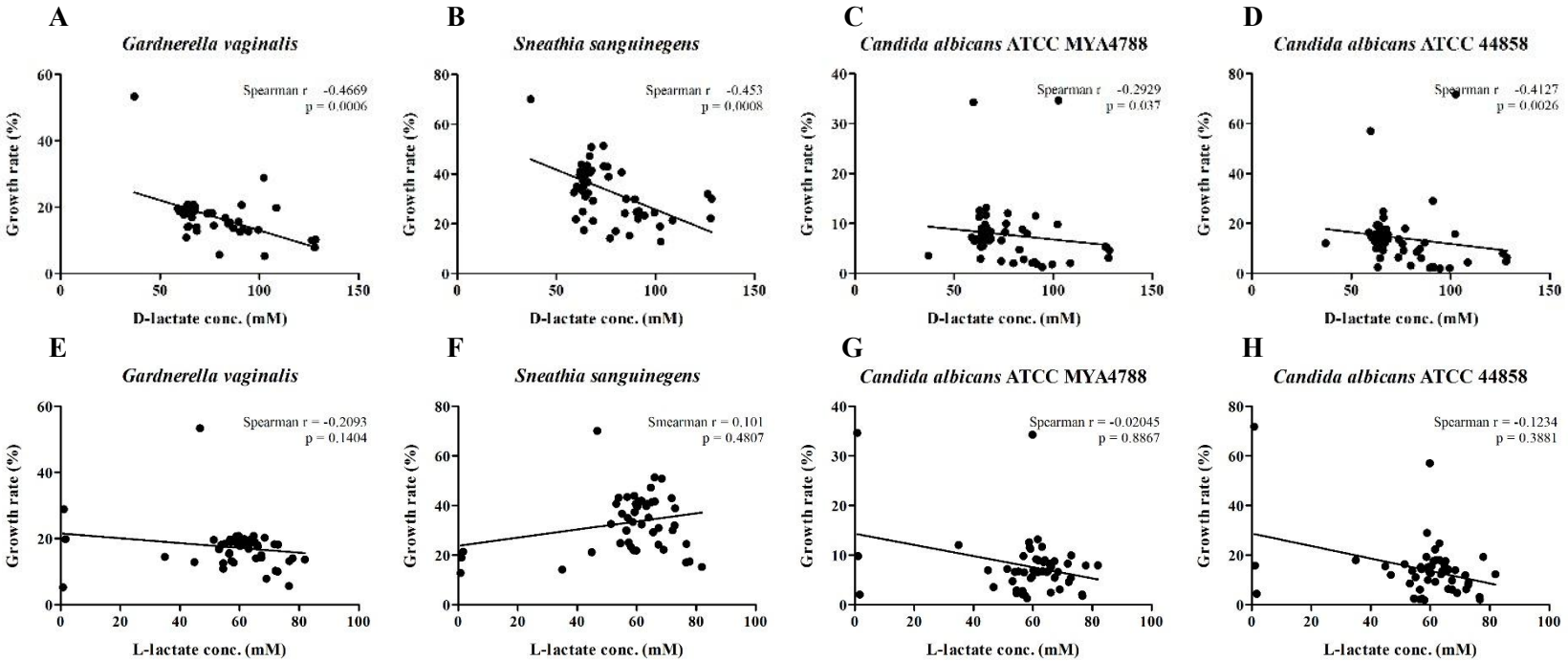


Figure 8. The correlation between lactate isomers produced by *Lactobacillus* spp. and growth rate of vaginal pathogens

Linear regression was performed to analyze the correlation of (A~D) D-lactate concentration and (E~H) L-lactate concentration with the growth rate of each pathogen (A, E; *G. vaginalis*, B, F; *S. sanguinegnes* and C, G; *C. albicans* ATCC MYA4788 and D, H; ATCC 44858). Each point represents a vaginal *Lactobacillus* strain. Spearman r and *P*-value were measured.

8. Antimicrobial activity of lactic acid on vaginal pathogens

To identify that lactic acid itself inhibit the growth of vaginal pathogens, solutions of D- or L-lactic acid were treated to vaginal pathogens at different concentrations (control, 5 mM, 50 mM, 75 mM, 100 mM and 200 mM). BV and VVC pathogens were suppressed their growth rate by both lactic acid isomers in a concentration dependent manner, but more effectively inhibited by D-lactic acid in general (Figure 9). The antimicrobial activity on *G. vaginalis* and *S. sanguinegens* was significantly different between the lactate isomers at concentrations from 5 mM to 100 mM ($P < 0.05$, Figure 9). *C. albicans* ATCC 44858 also was more effectively inhibited by D- lactic acid at concentrations over 50 mM, but *C. albicans* ATCC MYA4788 showed significant differences at only 50 mM (data not shown).

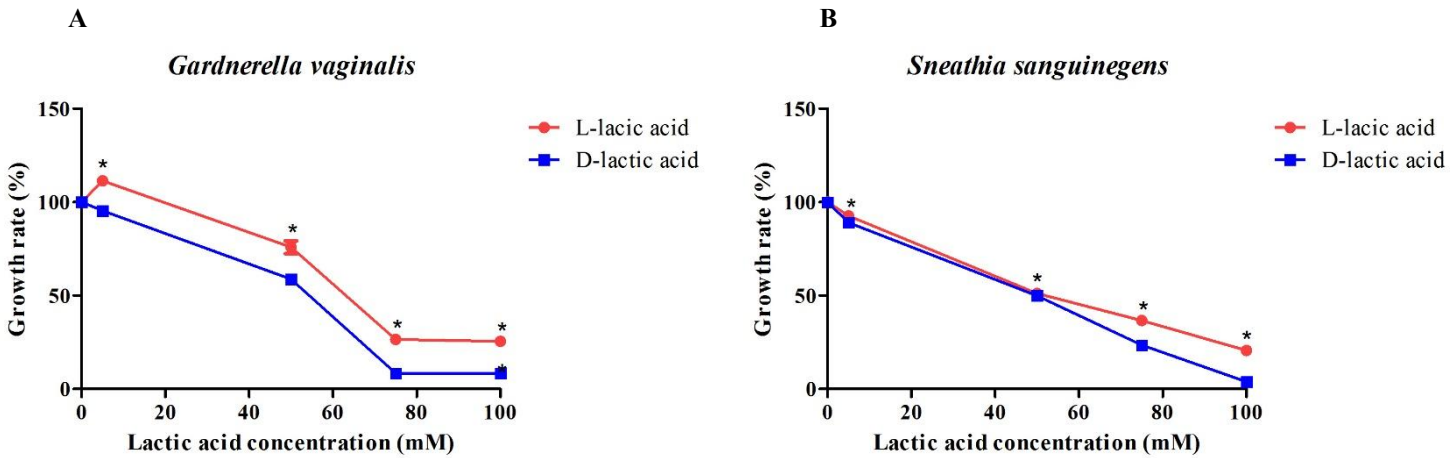


Figure 9. Inhibitory effect of each lactic acid isomer on vaginal pathogens

To clarify that each lactic acid isomer affects to vaginal pathogens differently, vaginal pathogens were treated by D- and L-lactic acid at different concentration (control, 5 mM, 50 mM, 75 mM, 100 mM and 200 mM). After incubation for 24 h, the growth rate of (A) *G. vaginalis*, (B) *Sneathia sanguingens* was measured by absorbance at 600 nm. Statistical significance for difference between isomers affecting the growth was analyzed by the Mann-Whitney test. * $P<0.05$.

9. Lactate productivity of *Lactobacillus* spp. by their origins

To compare vaginal *Lactobacillus* isolates with non-vaginal *Lactobacillus* isolates in terms of lactate productivity, the concentrations of D- and L-lactate were measured in the CFS of 33 gut-originated *Lactobacillus* strains (Figure 10). While all vagina-originated strains produce both lactate isomers, about 30 percent of strains isolated from the gut produced L-lactate only. Moreover, all strains belonging to *L. salivarius* not produced D-lactate. In comparison between the concentrations of the lactate isomers, vagina-originated strains produced significantly more D-lactate than L-lactate, but gut strains produced more L-lactate than D-lactate, showing the reverse producing patterns ($P < 0.0001$, Figure 10).

The ratio of D- to L-lactate was also calculated by dividing the D-lactate concentration by the L-lactate concentration to compare the lactate productivity by their origins. All strains isolated from the vagina or gut were compared in respect to the ratio of D- to L-lactate. The strains isolated from the vagina showed significantly higher D-/L-lactate ratio than strains isolated from the gut ($P < 0.0001$, Figure 11A). Furthermore, to confirm that the differences in D-/L-lactate ratio between their origins caused by not only different composition of the

species, the ratio of lactate isomers produced by *L. fermentum* and *L. gasseri* was compared between their origins. Although the species were commonly found in both tissues, they also showed significantly different D-/L-lactate ratio by their origins. ($P < 0.0001$, $P < 0.05$, Figure 11B~C).

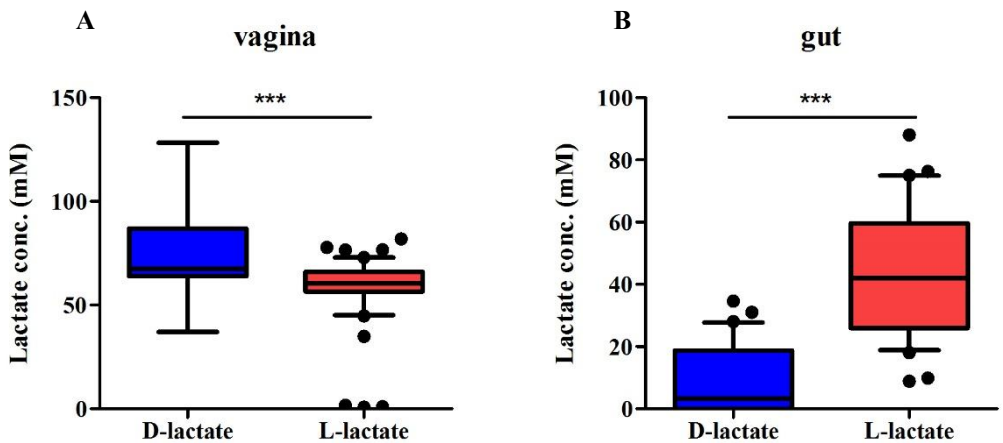


Figure 10. The concentration of D- and L-lactate produced by *Lactobacillus* spp. isolated from the vagina or gut

D- and L-lactate concentration produced by *Lactobacillus* strains isolated from (A) the vagina (n =51 strains) and (B) gut (n =33 strains) were measured. The whiskers of each boxplot represent the 10 to 90 percentiles. Statistical significance was measured by unpaired t-test. *** P<0.001

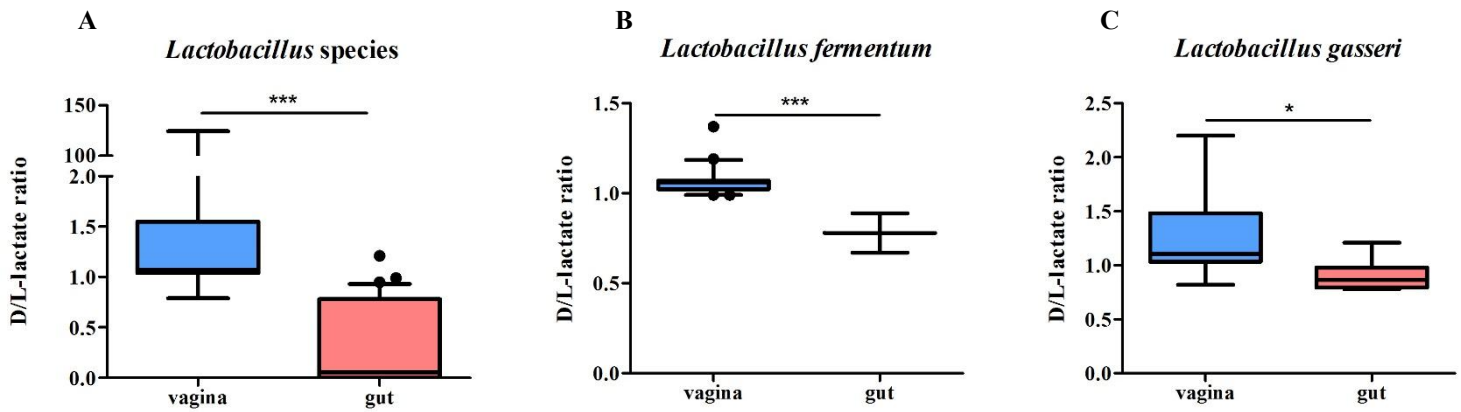


Figure 11. The ratio of D- to L-lactate produced by *Lactobacillus* spp. isolated from the vagina or gut

D-/L-lactate ratio was calculated by dividing the D-lactate concentration by the L-lactate concentration to compare the ratios by their origins. The ratio were carried out in (A) total *Lactobacillus* species (n= 51 and 33 strains for each origin), (B) *L. fermentum* (n= 13 and 2 strains) or (C) *L. gasseri* (n= 7 and 9 strains) species. The whiskers of each boxplot represent the 10 to 90 percentiles. Statistical significance was analyzed by the Mann-Whitney test. * $P<0.05$, *** $P<0.001$

10. Carbohydrate fermentation profile of *Lactobacillus* spp. by their origins

To assess qualitative carbohydrate fermentation profile of *Lactobacillus* strains by their origins, API test was conducted in randomly selected 49 strains isolated from the vagina or gut. The results are shown in yellow or blue color and each means fermentable or non-fermentable carbohydrate by treated *Lactobacillus* strain (Figure 12). Clustering analysis by carbohydrate and *Lactobacillus* strains was conducted based on the Euclidean distance. Among 49 carbohydrates, although most of them were fermented or unfermented by all tested strains, amidon starch and glycogen were not fermented by almost all of gut originated strains. Conversely, almost all of *Lactobacillus* strains isolated from the vagina not fermented methyl-alpha D-glucopyranoside, D-furanose, D-mannitol, D-melezitose, dulcitol, L-rhamnose, L-fucose, L-sorbose and D-sorbitol.

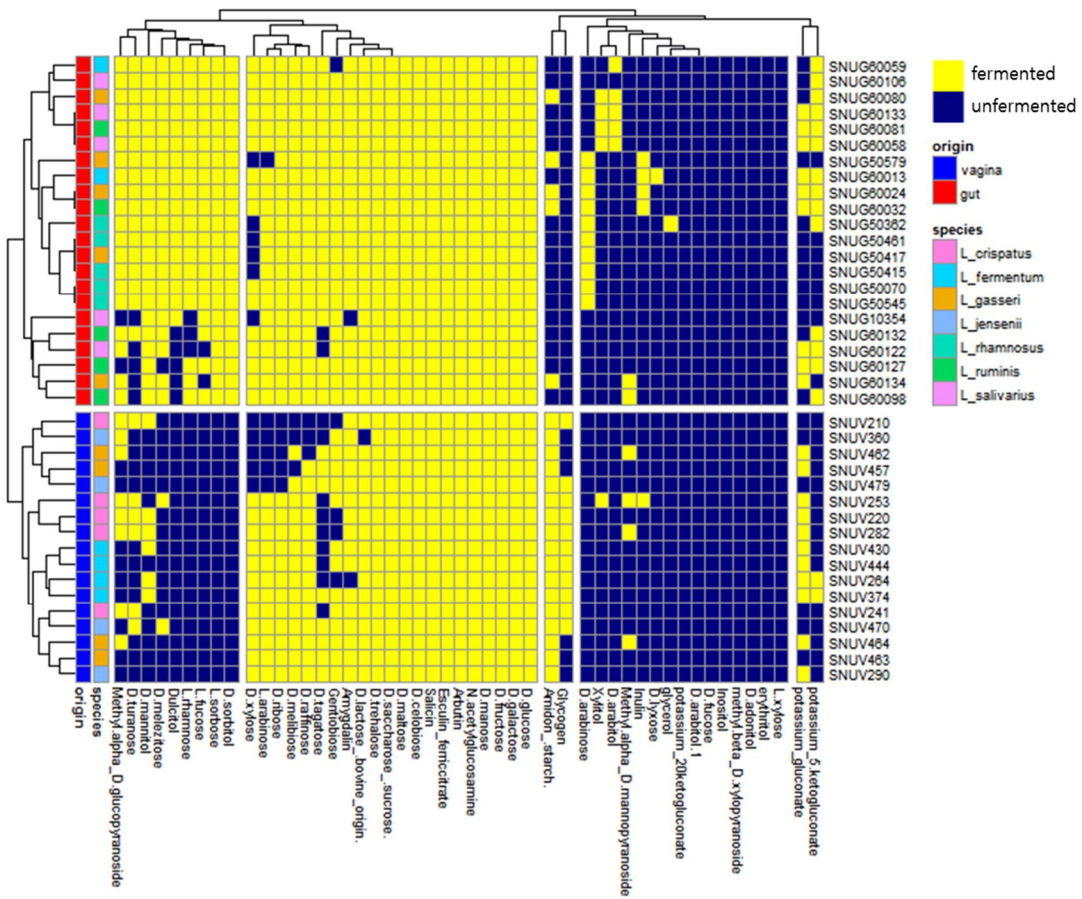


Figure 12. Carbohydrate fermentation profile of *Lactobacillus* spp. isolated from the vagina or gut

The fermentation profiles were generated based on API test. API was conducted in randomly selected strains (17 and 22 strains selected among lactobacilli isolated from the vagina and gut respectively). Yellow and blue color represent fermented and unfermented carbohydrate by lactobacilli.

IV. Discussion

Lactobacillus spp. characterize the majority of healthy vaginal microbiota and protect the host from a variety of infectious diseases including BV and VVC by producing a variety of metabolites, but their roles in health and diseases are still unclear. Our antimicrobial assay show that *Lactobacillus* spp. affect both the pathogenic bacteria and yeast. 51 *Lactobacillus* strains isolated from the vagina are used in this study and they are divided into four clusters by their inhibitory patterns against vaginal pathogens, *G. vaginalis*, *S. sanguinegens* and *C. albicans*. *Lactobacillus* strains belonging to Cluster Ⅳ inhibit the growth of all vaginal pathogens most effectively (Figure 6).

In the vaginal environment, while L-lactate is a metabolic product by vaginal epithelial cells and residential bacteria, D-lactate is mostly produced by vaginal microbiota (29, 37). Therefore the concentration of each lactate isomer and the ratio of D- to L-lactate in the vagina depend on the microorganisms which are in the vagina. Women whose vaginal microbiota were dominated by *L. crispatus* contain significantly higher D-lactic acid concentration in the vagina than those dominated by *L. iners* or *Gardnerella* spp.. Also, D-lactic acid exceed the level of L-lactic acid, as evidenced by the ratio of D- to L-lactic acid

being greater than 2, when *L. crispatus* was dominant (29). Conversely, women with BV showed a decreased level in the ratio of D- to L-lactic acid (31).

In our *in vitro* assay, the concentration of L-and D-lactate not differs based on the *Lactobacillus* species, but the ratio of D- to L-lactate is significantly higher in *L. crispatus* than *L. fermentum* (Figure 3). All of *L. fermentum* strains belong to Cluster Ⅲ and the majority of *L. crispatus* belong to Cluster Ⅳ showing the greatest effect on pathogenic growth inhibition. Also, the strains included in Cluster Ⅳ produced more D-lactate and showed higher D-/L-lactate ratio than the strains belonging to Cluster Ⅲ (Figure 7). Moreover, *Lactobacillus* species associated with both clusters, *L. crispatus* and *L. jensenii*, also showed same patterns in the lactate isomer production between clusters (Figure 7). Furthermore, our correlation analysis clarifies the association between the concentration of the lactate isomers and pathogenic inhibitory effects. The growth rates of tested vaginal pathogens have statistical negative correlation with D-lactate concentration and D-/L-lactate ratio produced by *Lactobacillus* strains (Figure 8). In other words, *Lactobacillus* strains that produce D-lactate more, inhibit the growth of vaginal pathogens better. Moreover, when

we treated the solutions of D- and L-lactic acid to vaginal pathogens, the growth rate is decreased by both isomers concentration-dependently but the levels of inhibited growth by L-lactic acid are significantly lower than D-lactic acid (Figure 9). This suggest that D-lactate concentration and D-/L-lactate ratio are the key characteristics of *Lactobacillus* strain associated with inhibitory effect rather than L-lactate concentration.

Lactobacillus spp. are found in various body tissues as well as vagina (38). Therefore, to investigate that lactate isomer productivity is species- or origin-specific, we compared the concentration of D- and L-lactate and D-/L-lactate ratio between vagina- and gut-originated *Lactobacillus* strains. The previous study showed that both *L. gasseri* and *L. crispatus* produce both lactate isomers but some *Lactobacillus* spp. produced only D-lactate or L-lactate (29). In our study, all strains isolated from the vagina produced both isomers, but only 70 percent of strains isolated from the gut are D-lactate producible and among the gut-originated strains, all strains belonging *L. salivarius* not produced D-lactate. Moreover, *Lactobacillus* strains isolated from the vagina produce more D-lactate than L-lactate significantly, but gut originated strains show reverse production between lactate isomers (Figure 10). When compared *L. fermentum* and *L. gasseri* between the origins, the ratio of D- to L-lactate is higher in the strains isolated from the vagina

than gut, in concordance with the comparison in all isolated strains between the origins (Figure 11). This suggests that the lactate isomers are produced in species- and origin- specific manner. Although vagina is close to anus, the number of species is much lower in vagina than gut. Approximately 50 species are in vagina, but more than 800 species in gut. The reason for these differences is still unclear, but may involve different nutrient availability (38). Nutrients existed in originated environment also may affect to the residential bacteria in terms of producing metabolites. Our API assay also show that *Lactobacillus* strains isolated from the vagina and gut ferment different carbohydrate (Figure 12). Furthermore, *Lactobacillus* strains isolated from the vagina have the both gene sequences coding D- and L-lactate dehydrogenase, but about fifty percent of gut-originated strains have L-lactate dehydrogenase only (Table S2). Even if the nucleotide sequences of lactate dehydrogenase coding regions are not differentiated between vagina- and gut-originated strains (Figure S2), this result may support that the fermentation characteristics of *Lactobacillus* strains differ by their origin. As a results, this suggests that *Lactobacillus* strains isolated from the vagina may affect to vaginal pathogens more than gut-originated *Lactobacillus* strains even if they are genetically same at species level.

There are some strengths on present study. First, we tested antimicrobial activity on the different four vaginal pathogens. *G. vaginails* and *S. sanguinegens* are vaginal pathogenic bacteria associated with BV and *C. albicans* ATCC MYA4788 and ATCC 44858 are the yeasts causing VVC. We confirmed that *Lactobacillus* strains affect to each pathogens differently, but generally they have similar patterns on pathogenic bacteria and yeasts. Second, we determined the most important factors affecting to the inhibitory effects on vaginal pathogens through our *in vitro* assay. Through the antimicrobial assay and correlation analysis, D-lactate concentration and D-/L-lactate ratio are selected the most important characteristics of *Lactobacillus* strains among the different characteristics including pH, hydrogen peroxide and L-lactate concentration in terms of antimicrobial activity. Third, we compared the lactate productivity between the vagina- and gut-originated strains. In comparison of the concentration of each lactate isomer and D-/L-lactate ratio, we identified that D-lactate and L-lactate are produced in species- and origin-specific manner and that *Lactobacillus* strains isolated from the vagina are more effectively usable to maintain vaginal health than strains isolated from the gut.

To our knowledge, this is the first *in vitro* study to explore the association between D-lactate concentration and inhibitory effect. We

found that D-lactate concentration and D-/L-lactate ratio are negatively associated with the growth rate of vaginal pathogens regardless of pathogenic bacteria and yeasts. Although the mechanisms that *Lactobacillus* spp. maintain healthy vaginal environment and protect the host from vaginal pathogens are still unclear, understanding the characteristics of production of D-lactate may lead to a better controlling of vaginal health and protecting from infectious diseases.

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VI. Supporting information

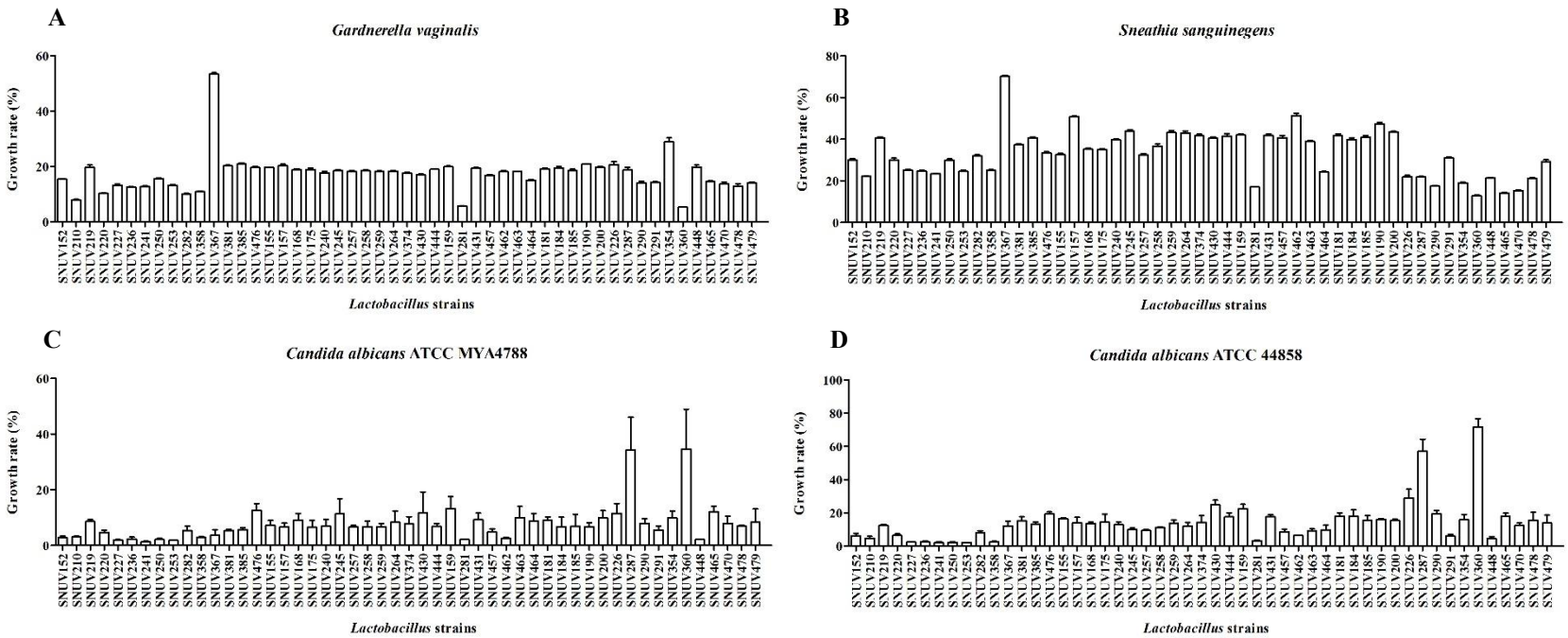

Figure S1. Antimicrobial activities of *Lactobacillus* spp. isolated from the vagina against vaginal pathogens

To determine effect of lactobacilli isolated from the vagina on each vaginal pathogens, BV pathogens (*G. vaginalis* and *S. sanginegens*) and VVC pathogens *(C. albicans*) were treated with *Lactobacillus* CFS and incubated for 24 h. The relative growth rate of (A) *G. vaginalis* KCTC 5096, (B) *S. sanguinegens*, (C) *C. albicans* ATCC MYA4788 and (D) *C. albicans* ATCC 44858 were calculated by absorbance at 600 nm.

Inhibition cluster	Species	Strain	Concentration (mM) D-lactate	L-lactate	D-/L-lactate
Cluster Ⅰ	*L. jensenii*	SNUV287	59.67	59.85	1.00
		SNUV360	102.63	0.82	124.49
Cluster Ⅱ	*L. crispatus*	SNUV367	37.05	46.75	0.79
Cluster Ⅲ	*L. crispatus*	SNUV219	67.02	63.63	1.05
		SNUV381	63.39	59.30	1.07
		SNUV385	63.89	59.69	1.07
		SNUV476	62.75	58.69	1.07
	L. fermentum	SNUV155	58.88	51.36	1.15
		SNUV157	67.52	68.40	0.99
		SNUV168	63.77	64.00	1.00
		SNUV175	60.26	57.04	1.06
		SNUV240	61.90	60.21	1.03
		SNUV245	62.54	59.18	1.06
		SNUV257	65.94	61.68	1.07
		SNUV258	65.70	55.11	1.19
		SNUV259	73.90	53.92	1.37
		SNUV264	75.68	71.73	1.06
		SNUV374	65.50	66.08	0.99
		SNUV430	66.05	63.11	1.05
		SNUV444	67.69	65.16	1.04
	L. gasseri	SNUV159	66.17	61.62	1.07
		SNUV431	66.32	61.07	1.09
		SNUV457	82.91	53.16	1.56
		SNUV462	73.72	66.05	1.12
		SNUV463	76.32	72.92	1.05
	L. jensenii	SNUV181	64.24	61.83	1.04
Cluster Ⅲ *(continued)*	*L. jensenii (continued)*	SNUV184	64.76	63.21	1.02
		SNUV185	61.90	60.43	1.02
		SNUV190	66.78	64.76	1.03
		SNUV200	65.53	56.82	1.15
Cluster Ⅳ	*L. crispatus*	SNUV152	85.10	56.55	1.50
		SNUV210	127.86	69.04	1.85
		SNUV220	128.30	72.09	1.78
		SNUV227	91.63	57.28	1.60
		SNUV236	90.37	54.53	1.66
		SNUV241	94.47	58.02	1.63
		SNUV250	89.58	56.58	1.58
		SNUV253	99.44	76.67	1.30
		SNUV282	126.31	72.73	1.74
		SNUV358	63.27	54.50	1.16
	L. gasseri	SNUV281	79.89	76.58	1.04
		SNUV464	84.46	67.33	1.25
	L. jensenii	SNUV226	91.13	58.93	1.55
		SNUV290	63.86	77.80	0.82
		SNUV291	64.50	67.36	0.96
		SNUV354	102.19	1.07	95.63
		SNUV448	108.55	1.65	65.83
		SNUV465	77.06	34.99	2.20
		SNUV470	86.89	81.89	1.06
		SNUV478	68.48	44.85	1.53
		SNUV479	68.45	65.50	1.05

Table S1. *Lactobacillus* species and strains belonging to each inhibition cluster

Vaginal *Lactobacillus* strains were divided into four clusters based on their inhibitory patterns by the Euclidean method

Lactobacillus species	Strain designation	Origin	No. of copies per genome[a]			IMG Genome ID
			DLD	LLD	LAR	
L. acidophilus	La-14	gut	0	1		2563366581
L. acidophilus	NCFM	gut	0	1		637000138
L. crispatus	EM-LC1	gut	1	3		2576861664
L. fermentum	ATCC 14931	gut	3	5		643886061
L. gallinarum	HFD4	gut	0	2		2648501845
L. gasseri	130918	gut	0	1		2630968908
L. gasseri	ATCC 33323	gut	0	1		639633030
L. johnsonii	NCC 533	gut	0	1		637000140
L. paracasei	8700:2	gut	0	3		643886139
L. plantarum	HFC8	gut	2	1	1	2654587681
L. plantarum	ZJ316	gut	2	1	1	2561511196
L. reuteri	ATCC 55730	gut	3	4		650716048
L. salivarius	UCC118	gut	2	1		637000143
L. crispatus	125-2-CHN	vagina	1	3		647533176
L. crispatus	2029	vagina	1	3		2602041603
L. crispatus	214-1	vagina	1	3		647000264
L. crispatus	CTV-05	vagina	1	2		651285003
L. crispatus	JV-V01	vagina	1	2		643886212
L. crispatus	MV-1A-US	vagina	1	2		646206277
L. crispatus	MV-3A-US	vagina	1	3		647533177
L. crispatus	ST1	vagina	1	3		646564539
L. gasseri	202-4	vagina	1	3		645058712
L. gasseri	224-1	vagina	1	3		647000265
L. gasseri	JV-V03	vagina	1	3		2562617156
L. gasseri	MV-22	vagina	1	2		2562617170
L. jensneii	115-3-CHN	vagina	2	1		647533179
L. jensneii	269-3	vagina	2	1		645058722
L. jensneii	JV-V16	vagina	2	1		648276683
L. jensneii	SJ-7A-US	vagina	2	1		647533180
L. plantarum	CMPG5300	vagina	3	1	1	2667527570

Table S2. Table of nucleotide sequences of lactate dehydrogenase coding regions of *Lactobacillus* spp.

[a] LLD, l-lactate dehydrogenase; DLD, d-lactate dehydrogenase; LAR, Nickel-dependent lactate racemase

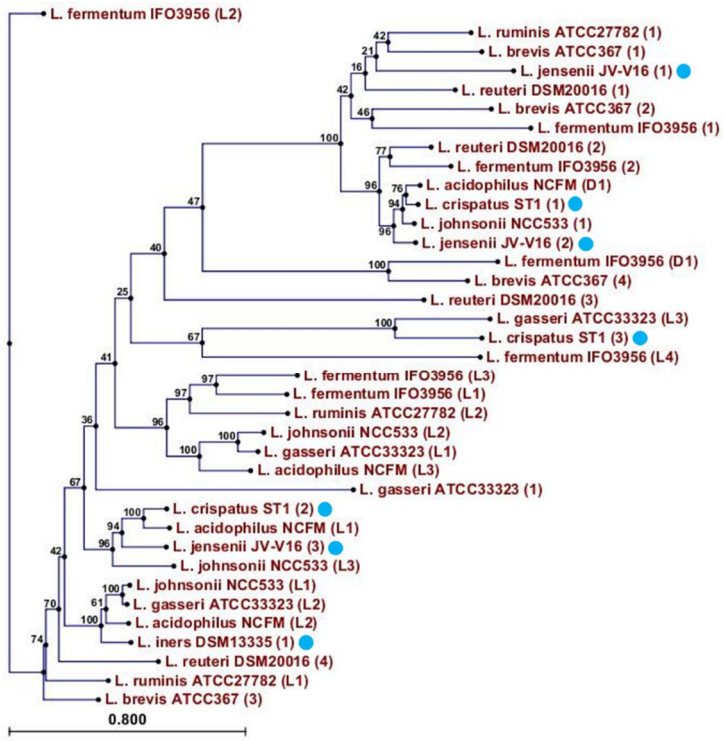

Figure S2. Alignment tree of nucleotide sequences of lactate dehydrogenase coding regions of *Lactobacillus* spp.

The gene sequences were obtained from NCBI and used to determine the differences in sequence between their origins. Blue dot means that the strains are originated from the vagina and the remainder are gut-originated strains. In parentheses, D or L express that the sequence is coding D- or L-lactate dehydrogenase. If a strain have over two copies of the sequence, they are numbered and also expressed in parentheses.

국문초록

건강한 여성의 질 내 균총에서 유래한 락토바실러스 균주의 질염 병원균 저해효과 연구

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락토바실러스는 락틱애씨드, 과산화수소, 박테리오신 등을 생성하며 건강한 질 내 균총에서 중요한 역할을 하고 있다. 특히 질 내 락토바실러스가 감소하고 병원균이 증식하는 현상은 세균성 질염이나 칸디다증과 같은 다양한 질병 발생과 관련이 있는 것으로 알려져 있다. 가드네렐라는 질염을 유발하는 대표적인 병원성 세균이며, 스니치아 속의 세균은 임신중독증, 유산, 조산, 자궁경부암 등 다양한 증상과 관련된 기회감염균으로써 최근 그에 대한 관심이 증가하고 있다. 이 연구에서는 건강한 여성의 질에서 분리한 51개 락토바실러스 균주를 이용하여 세균성 질염과 관련된 병원성 세균과 칸디다증의 원인이 되는 효모의 성장 저해효과를 확인하고, 이러한 저해효과와 락토바실러스의 생산물 사이의 상관관계를 평가하였다. 락토바실러스는 병원체 저해패턴에 따라 네 개의 클러스터로 구분되었으며, 가장 강한 저해효과를 나타내는 네 번째 클러스터에 속한 균주가 다른 균주에 비해 D-락틱애씨드 이성질체 생산 비율이 높은 것으로 나타났다. 이를 바탕으로 락토바실러스의 락틱애씨드 이성질체 생산성과 병원체 저해능력간의 상관관계를 명확히 하기 위해 상관분석을 수행한 결과, 락토바실러스의 D-락틱애씨드 생성 정도가 병원체의 성장률과 유의한 음의 상관관계를 갖는 것을 확인하였다. 반면, L-락틱애씨드, PH, 과산화수소 생산성 등 다른 요인들은 병원체의 성장률과 유의한 상관관계를 나타내지 않았다. 또한 질 유래 락토바실러스 균주의 락틱애씨드 이성질체 생산성을 장에서 분리한 락토바실러스 균주의 생산성과 비교하였을 때, 질 유래 균주의 D-락틱애씨드 생산성이 장 유래 균주보다 유의하게 높았다. 따라서 이 연구에서는 락토바실러

스가 종과 기원에 따라 특이적으로 락틱애씨드 이성질체를 생산하며, 특히 락토바실러스의 D-락티애씨드 생산능력이 락토바실러스의 질염 병원체 저해효과를 결정한다는 것을 확인할 수 있었다.

주요 단어: 락토바실러스, 가드네렐라, 스니치아, 칸디다, 세균성 질염, 칸디다성 질염, 칸디다증

학번: 2015-24056